ALDEHYDE OXIDASE GENE DERIVED FROM PLANT AND UTILIZATION THEREOF

FIELD OF THE INVENTION

The present invention relates to an aldehyde oxidase gene derived from a plant and utilization thereof.

It has been known that a natural plant growth hormone auxin alternatively IAA or indoleacetic acid is produced from tryptophane via indoleacetaldehyde followed by the action of an oxidase in higher plants. The hormone is deeply involved in various morphogenesis and environmental adaptation of a plant by its physiological activity and has significant effects on maturing by growth acceleration in general crop cultivation, improvement in yield and in quality by rooting acceleration in nursery plant production, increase in yield by growth acceleration of fruits in fruit vegetable cultivation, increase in added value by acceleration of flowering and elongation of life by prevention of defoliation or aging in ornamental plant cultivation. Therefore, there has been a strong demand for a method for artificially controlling the said enzyme for industry and particularly agricultural production.

Under these circumstances, the present inventors have successfully determined the total amino acid sequence and gene of the enzyme and completed the present invention.

Thus, the present invention provides:

1) An aldehyde oxidase gene which is a 4.4 Kbp gene obtainable from a plant and which encodes an amino acid sequence of an enzyme capable of oxidizing an aldehyde compound to a carboxylic acid (hereinafter, referred to as the gene of the present invention),

2) The aldehyde oxidase gene according to item 1), wherein the aldehyde compound is indoleacetaldehyde and the carboxylic acid is indoleacetic acid,

3) The aldehyde oxidase gene according to item 1 or 2 which is derived from a maize plant (*Zea mays L.*),

4) The aldehyde oxidase gene according to item 1 which is a nucleotide sequence encoding an amino acid sequence shown by SEQ ID NO: ~~1~~ 2,

5) The aldehyde oxidase gene according to item 4 which has a nucleotide sequence shown by SEQ ID NO: ~~2~~ 1 (loci of CDS being 46..4120),

6) The aldehyde oxidase gene according to item 1 which is a nucleotide sequence encoding an amino acid sequence shown by SEQ ID NO: ~~3~~ 4,

7) The aldehyde oxidase gene according to item 6 which has a nucleotide sequence shown by SEQ ID NO: ~~4~~ 3 (loci of CDS being 91..4138),

8) A plasmid comprising the aldehyde oxidase gene according to item 1, 2, 3, 4, 5, 6 or 7,

9) A transformant transformed by introducing the plasmid according to item 8 into a host cell,

10) The transformant according to item 9, wherein the host cell is a microorganism,

11) The transformant according to item 9, wherein the host cell is a plant,

12) A process for constructing an expression plasmid which comprises ligating:

(1) a promoter capable of functioning in a plant cell,

(2) an aldehyde oxidase gene according to item 1, 2, 3, 4, 5, 6 or 7 and

(3) a terminator capable of functioning in a plant in a functional manner and in the said order described above,

13) An expression plasmid comprising:

(1) a promoter capable of functioning in a plant cell,

(2) an aldehyde oxidase gene according to item 1, 2, 3, 4, 5, 6 or 7 and

(3) a terminator capable of functioning in a plant which are ligated in a functional manner and in the said order described above,

14) A process for controlling production of an aldehyde oxidase in a transformant which comprises introducing, into a host cell, an expression plasmid comprising:

(1) a promoter capable of functioning in a plant cell,

(2) an aldehyde oxidase gene and

(3) a terminator capable of functioning in a plant which are ligated in a functional manner and in the said order described above to transform said host cell,

15) The process according to item 14, wherein the aldehyde oxidase gene is derived from a plant and the host cell is a plant, and

16) The process according to item 14, wherein the expression plasmid is the expression plasmid according to item 13.

EMBODIMENTS OF THE INVENTION

The present invention will be described in more detail.

The gene of the present invention comprises about 4.4 kbp nucleotide obtainable from a plant and is an aldehyde oxidase gene that encodes an amino acid sequence of an enzyme capable of oxidizing an aldehyde compound to generate a carboxylic acid. For example, it is capable of oxidizing indoleacetaldehyde to generate indoleacetic acid.

The gene of the present invention can be obtained from a plant, for example, maize or the like. The gene of the present invention and the enzyme as the translation product of it have an action of oxidizing an acetaldehyde compound to a carboxylic acid in a cell. Said enzyme may also act, for example, on benzaldehyde, butyraldehyde, protocatechualdehyde or the like as the substrate, in

addition to indolealdehyde. Of course, a single enzyme may act on plural compounds as substrates.

The gene of the present invention specifically includes, for example, a gene which is a nucleotide sequence encoding an amino acid sequence shown by SEQ ID NO: ~~1~~ 2 and a gene which is a nucleotide sequence encoding an amino acid sequence shown by SEQ ID NO: ~~2~~ 4 as well as an equivalent of them. The expression "an equivalent of them" used herein means an aldehyde oxidase gene having a nucleotide sequence of an aldehyde oxidase gene that encodes an amino acid sequence shown by SEQ ID NO: ~~1~~ 2 or SEQ ID NO: ~~2~~ 4 with a single nucleotide or plural nucleotides added, deleted or replaced, and refers to a DNA which is an analog having the same function. More particularly, this includes a gene having a nucleotide sequence shown by SEQ ID NO: ~~2~~ 1 (loci of CDS being 46..4120) or a nucleotide sequence shown by SEQ ID NO: ~~4~~ 3 (loci of CDS being 91..4138).

The gene of the present invention can be obtained by the following process.

For example, seeds of Golden Cross Bantam 70 (purchased from Sakata-no-tane), a maize cultivar, are subjected to a treatment for hastening of germination by immersing overnight in running tap water, subsequently seeded on a paper towel moistened with water and placed in red light (0.8 W/m^2)

under a condition of 25℃ for 2 days and then in the dark for 1 day to allow germination. Top portions of young sheaths grown to 1.0 - 1.5 cm from the obtained seedlings are excised under a green safety light, immediately frozen with liquid nitrogen and stored at -30℃ as samples for purification of enzymes and samples for extracting RNAs.

For purifying aldehyde oxidase from the frozen samples prepared in this manner, it is appropriate to use a method described in T. Koshiba et al., Plant Physiology, 1996, 110, 781 - 789.

In order to prevent decrease in activity of the enzyme and decomposition of the protein during procedures of extraction and purification, it is preferred to carry out all the treatments in the purification steps at a lower temperature of 2 - 4℃, as is ordinary manner in such procedures. First, 150 - 200 g of the frozen sample is taken as a material for one batch of purification. The material is mechanically crushed by a homogenizer or the like with addition of 400 ml of 0.1 M phosphate buffer (pH 7.4) and centrifuged at 12,000 g for 30 minutes. The supernatant is separated as a crude enzyme standard sample. From the crude enzyme standard sample, a fraction is obtained with 30 - 50% saturated ammonium sulfate, dialyzed against 20 mM Tris HCl buffer (pH 8.0) and centrifuged at 20,000 g for 20 minutes. The supernatant from centrifugation is passed over an

ion-exchange column (for example, DEAE TOYOPEARL 650 M, manufactured by Tosoh) and a fraction with an aldehyde oxidase activity is collected. Said fraction with the specific activity is subjected to chromatography with a hydrophobic column, a hydroxyapatite column and an ion-exchange column (for example, DEAE-5PM) in this order and purified until the fraction with aldehyde oxidase activity is detected as an almost single protein band by silver staining after electrophoresis.

According to the above described purification procedure, about 2,000 times purification, in terms of the amount of protein in the crude enzyme standard sample, is usually possible. It can be confirmed that the finally purified protein has a size of about 300 kD in molecular weight by the gel filtration column process. Further, it can be detected as a band having a size of about 150 kD in molecular weight by SDS polyacrylamide gel electrophoresis (SDS-PAGE), indicating that said enzyme forms a dimer.

In the above described fractionating process by column chromatography, effective collection of the fraction with aldehyde oxidase activity can be achieved making use of measurement of aldehyde oxidase activity in respective fractions. For this purpose, a method in which indoleacetaldehyde is added to the purified fraction as a substrate and the amount of produced indoleacetic acid is

determined by HPLC, for example, can be utilized. Precisely, 100 μl of reaction solution consisting of 5 - 50 μl of the purified fraction, 0.1 mM indoleacetaldehyde and 0.1 mM phosphate buffer (pH 7.4) is prepared. The solution is incubated at 30℃ for 30 minutes to effect the reaction and, immediately after, 8 μl of 1 N HCl, 5 μl of 2.0 M sodium hydrogen sulfite and 50 μl of methanol are added to the solution to quench the reaction. The reaction solution is centrifuged at 15,000 g for 5 minutes and 100 μl of the obtained supernatant is taken as a analytical sample for HPLC. By detecting absorption at 280 nm, indoleacetaldehyde as the substrate and indoleacetic acid as the reaction product can be quantitatively analyzed. It is effective to carry out HPLC with, for example, ODS C18 column and to elute with 20 - 50% linear gradient of methanol containing 0.1% acetic acid.

The protein obtained in this manner is partially digested and the digested peptide is analyzed to obtain a partial amino acid sequence information. Usually, the purified aldehyde oxidase sample is separated by SDS-PAGE and a protein band of 150 kD is collected by excision. The collected gel fragments are treated, for example, with Achromobacter Protease I (API) in the presence of 0.1% SDS and digested peptide fragments are extracted. This is loaded, for example, on a reverse phase HPLC accompanied by a pre-column of an anion exchanger (DEAE) to separate peptides

and recover them. The amino acid sequences are determined by a protein sequencer and parts of the samples are subjected to molecular weight determination by MALDI-TOF to check accuracy of the obtained amino acid sequence information.

Then, an oligo DNA expected to encode the amino acid sequence is synthesized on the basis of the obtained amino acid sequence information. Further, RT-PCR is conducted using a total RNA as a template to amplify cDNA partial fragment, which is then cloned into a plasmid vector.

For extraction of the total RNA, 7 g of the frozen sample, for example, is triturated in liquid nitrogen with a mortar and a pestle to form fine powders. After evaporating liquid nitrogen, RNA is extracted by the conventional manner, for example, using guanidine thiocyanate/cesium chloride process and the total RNA is collected from the extract by ethanol precipitation. By this procedure, usually 1 mg of the total RNA is obtained.

For amplification of cDNA, a reverse transcription reaction is carried out using, among synthetic oligo DNAs, one synthesized in antisense orientation as a primer and binding it to a transcription product of a target RNA contained in the total RNA. The reverse transcription reaction can be conducted using a commercially available reverse transcription PCR kit, for example, RNA-PCR kit (manufactured by Perkin-Elmer Cetus Instruments). Then, the

obtained reverse transcription product can be subjected again to PCR in which an oligo DNA synthesized in sense orientation is added to amplify cDNA fragment.

The obtained cDNA amplification fragment is purified and cloned into a plasmid vector. As the plasmid vector, for example, pCRII (manufactured by Invitrogen) can be used and cDNA amplification fragment can be cloned by transforming *E. coli* according to the conventional manner and screening transformants having an insert. The nucleotide sequence of the clone is determined using, for example, ABI PRISM Dye Primer Cycle Sequencing Ready Reaction Kits (manufactured by Applied Biosystems) on the obtained cDNA clone.

Sense and antisense primers for part of nucleotide sequence in cDNA partial fragment obtained in this manner can be synthesized and subjected to RACE to obtain cDNA fragments having terminals in 5'-orientation and 3'-orientation, respectively. A complete length cDNA can be obtained by ligating them and cloning into a plasmid vector. For the RACE, a commercially available Marathon cDNA Amplification Kit (manufactured by Clontech), for example, can be used.

The gene of the present invention can be utilized in the following manner.

For example, a host cell such as a microorganism, a plant or the like is transformed by introducing the gene of the

present invention to form a transformant.

In order to introduce and express the gene of the present invention in a plant cell, an expression plasmid comprising (1) a promoter capable of functioning in a plant cell, (2) a gene of the present invention (an aldehyde oxidase gene described in items 1 to 7 above) and (3) a terminator capable of functioning in a plant cell which are ligated in a functional manner in a plant cell and in the said order described above and introduced in a plant cell to transform said cell.

The expression "in a functional manner" used herein means that, when the constructed plasmid is introduced into a plant cell to transform it, the gene of the present invention is integrated under the control of a promoter such that the gene is normally transcribed/translated and have a function of expressing a protein in said plant cell.

The promoter capable of functioning in a plant cell includes, for example, T-DNA derived constitutive type promoters such as nopaline synthase gene (NOS) promoter, octopine synthase gene (OCS) promoter and the like, plant virus derived promoters such as cauliflower mosaic virus (CaMV) derived 18S and 35S promoters and the like, and inducible type promoters such as phenylalanine ammonialyase (PAL) gene promoter, chalcone synthase (CHS) gene promoter, pathogen-related (PR) gene promoter and the like. Further,

it includes other known plant promoters.

The terminator capable of functioning in a plant cell includes, for example, T-DNA derived constitutive type terminators such as nopaline synthase gene (NOS) terminator and the like, plant virus derived terminators such as garlic virus GV1, GV2 terminators and the like. Further, it includes other known plant terminators.

For transforming a plant cell by introducing such plasmid into a plant cell, the above described expression plasmid is introduced into a plant cell by any of conventional means such as *Agrobacterium* infection method (JP-B-2-58917 and JP-A-60-70080), electroporation method into protoplast (JP-A-60-251887 and JP-A-5-68575), particle gun method (JP-A-508316 and JP-A-63-258525) and the like, and a transformed plant cell can be obtained by selecting a plant cell into which the gene of the present invention is introduced. The transformed plant is obtained by regenerating a plant according to a conventional plant cell culturing process, for example, described in Uchimiya, Manual for Plant Gene Manipulation (Method for Producing Transgenic Plants), Published by Kodansha Scientific (ISBN 4-06-153515-7 C3045), 1990, pages 27 - 55.

Further, the present invention provides a process for controlling production of an aldehyde oxidase in a transformant which comprises introducing, into a host cell,

an expression plasmid comprising (1) a promoter capable of functioning in a plant cell, (2) an aldehyde oxidase gene and (3) a terminator capable of functioning in a plant which are ligated in a functional manner and in the said order described above to transform said host cell.

The promoter capable of functioning in a plant cell includes, for example, lacZ gene promoter of lactose operon in *E. coli*, alcohol dehydrogenase gene (ADH) promoter in yeast, Adenovirus major late (Ad.ML) promoter, early promoter of SV 40, Baculovirus promoter and the like. When the host is a plant, promoters capable of functioning in a plant as described above may also be included.

The terminator capable of functioning in a plant cell includes, for example, HIS terminator sequence in yeast, ADHI terminator, early splicing region of SV 40 and the like. When the host is a plant, terminators capable of functioning in a plant as described above may also be included.

The aldehyde oxidase gene may be any one insofar as it is a gene encoding an amino acid sequence of an enzyme capable of oxidizing an aldehyde compound to form a carboxylic acid. This includes, for example, aldehyde oxidase genes derived from plants and preferably the gene of the present invention (an aldehyde oxidase gene described in items 1 to 7 above).

Transformation of a host cell by introducing such plasmid into said host cell can be effected by a method

generally used in the field of genetic engineering.

When the host cell is a plant cell, it can be effected, for example, by a method generally used in the field of plant genetic engineering and the field of plant tissue cultivation as described above.

The transformation of a plant by introducing the gene of the present invention may bring about enhancement of generally known physiological action of auxin or supression of the same. For example, by enhancing the activity of auxin through a sense gene, elongation growth and differentiation to vascular bundle of the host cell can be accelerated resulting in growth acceleration of a plant and enhanced capacity of storing assimilation products. As a result, early maturing of crops, enlargement of harvest such as fruits and improvement in yield or quality can be expected and realized. To the contrary, by suppressing the activity of auxin through a sense gene, spindly growth of a plant is prevented and a plant capable of growing under improper environmental conditions such as insufficient insolation can be bred. Further, by adequately controlling growth, dwarfing of crops becomes possible and application, for example, to prevention of lodging of rice plants and shortening of cut flowers become possible. As a result, improvement in yield and quality can be expected.

Addition of hormone to the medium is generally essential

for aseptic cultivation of cells or tissue of a plant. When auxin activity in a plant is enhanced by introducing and expressing the gene of the present invention thereby increasing production of aldehyde oxidase in a transformant, said plant is expected to be in a state in which capacity of cell proliferation, differentiation and individual regeneration in the sterile culture is enhanced. Therefore, it is possible to create a so-called easily cultured strain and this is useful in the production of nursery plant of virus-free crops for which tissue culture-nucleotide mass culture is conducted and garden crops such as flower and ornamental plants.

EXAMPLES

The present invention will now be described in more detail by means of Examples. It is to be understood, however, that the scope of the present invention is not limited to these Examples.

Example 1 (Preparation of maize young sheath)

Seeds of Golden Cross Bantam 70 (purchased from Sakata-no-tane), a maize cultivar, were subjected to a treatment for hastening of germination by immersing overnight in running tap water, subsequently seeded on a paper towel moistened with water and placed in red light (0.8 W/m^2)

under a condition of 25℃ for 2 days and then in the dark for 1 day to allow germination. Top portions (1.0 - 1.5 cm) of young sheaths grown from the obtained seedlings to 2 - 3 cm were excised under a green safety light, immediately frozen with liquid nitrogen and stored at -30℃.

Example 2 (Preparation of aldehyde oxidase)

All the procedures in the following purification steps were conducted at a low temperature of 2 - 4℃.

First, about 200 g of the frozen sample prepared in Example 1 was taken as a material for one batch of purification. The material was mechanically crushed by a homogenizer with addition of 400 ml of 0.1 M phosphate buffer (pH 7.4) and centrifuged at 12,000 g for 30 minutes. The supernatant was separated as a crude enzyme standard sample. Subsequently, from the crude enzyme standard sample, a fraction was obtained with 30 - 50% saturated ammonium sulfate, dialyzed against 20 mM Tris HCl buffer (pH 8.0) and centrifuged at 20,000 g for 20 minutes. The supernatant from centrifugation was passed over an ion-exchange column (DEAE TOYOPEARL 650 M, manufactured by Tosoh) and a fraction with an aldehyde oxidase activity was collected on the basis of activity measurement conducted in a manner described below in Example 3. Said fraction with activity was subjected to chromatography with a hydrophobic column, a hydroxyapatite column and an

ion-exchange column (DEAE-5PM) in this order and purified until the fraction with aldehyde oxidase activity was detected as an almost single protein band by silver staining on electrophoresis.

By the above described purification procedure, about 0.09 mg of protein was recovered from 1,873 mg of protein in the crude enzyme standard sample, and ratio of enzyme activity for aldehyde oxidase to the original was 1,950 times. It was confirmed that the finally purified protein had a size of about 300 kD in molecular weight by the gel filtration column process. Further, it was detected as a band having a size of about 150 kD in molecular weight by SDS polyacrylamide gel electrophoresis (SDS-PAGE), indicating that said enzyme formed a dimer.

Example 3 (Method for measuring aldehyde oxidase activity)

Measurement of aldehyde oxidase activity in the respective purified fractions described in Example 2 was carried out by a method in which indoleacetaldehyde was added to the purified fraction as a substrate and the amount of produced indoleacetic acid (IAA) was determined by HPLC. The reaction was carried out with 100 μl of reaction solution consisting of 5 - 50 μl of the purified fraction, 0.1 mM indoleacetaldehyde and 0.1 mM phosphate buffer (pH 7.4). The solution was incubated at 30℃ for 30 minutes and, immediately

after, 8 μl of 1 N HCl, 5 μl of 2.0 M sodium hydrogen sulfite and 50 μl of methanol were added to the solution to quench the reaction. The reaction solution was centrifuged at 15,000 g for 5 minutes and 100 μl of the obtained supernatant was taken as a analytical sample for HPLC. By detecting absorption at 280 nm, indoleacetaldehyde and indoleacetic acid were quantitatively analyzed. HPLC was carried out with ODS C18 column and eluted with 20 - 50% linear gradient of methanol containing 0.1% acetic acid.

Example 4 (Peptide digestion of aldehyde oxidase: partial amino acid sequence)

The purified protein obtained in Example 2 was separated by SDS-PAGE and a protein band of 150 kD was collected by excision. The collected gel fragments were reacted with Achromobacter Protease I (API) in the presence of 0.1% SDS and digested peptide fragments were extracted. This was passed over a reverse phase HPLC accompanied by a pre-column of an anion exchanger (DEAE) to separate peptides, which were collected. The amino acid sequences were determined by a protein sequencer (ABI 477A).

As a result, the following 4 sequences were obtained as the partial amino acid sequences.

The first one was a sequence, shown below, having 18 amino acid residues:

Gln Val Asn Asp Val Pro Ile Ala Ala Ser Gly Asp Gly Trp Tyr His Pro Lys

and it was confirmed that the sequence corresponded to Nos. 235 to 252 residues in the amino acid sequence shown by SEQ ID NO: ~~1~~ 2.

The second one was a sequence, shown below, having 16 amino acid residues:

Thr Asn Ser Asp Gly Leu Val Ile His Asp Gly Thr Trp Thr Tyr Lys

and it was confirmed that the sequence corresponded to 1,234 to 1,249 residues in the amino acid sequence shown by SEQ ID NO: ~~1~~ 2 or to 1,226 to 1,241 residues in the amino acid sequence shown by SEQ ID NO: ~~3~~ 4.

The third one was a sequence, shown below, having 20 amino acid residues:

Ser Ile Glu Glu Leu His Arg Leu Phe Asp Ser Ser Trp Phe Asp Asp Ser Ser Val Lys

and it was confirmed that the sequence corresponded to Nos. 253 to 272 residues in the amino acid sequence shown by SEQ ID NO: ~~1~~ 2.

The fourth one was a sequence, shown below, having 21 amino acid residues:

Val Gly Ala Glu Ile Gln Ala Ser Gly Glu Ala Val Tyr Val Asp Asp Ile Pro Ala Pro Lys

and it was confirmed that the sequence corresponded to Nos. 591 to 611 residues in the amino acid sequence shown by SEQ ID NO: ~~1~~ 2.

Parts of these digested peptide samples were subjected to molecular weight determination by MALDI-TOF to check accuracy of the obtained amino acid sequence.

Example 5 (Preparation of total RNA from maize young sheath and synthesis of cDNA)

In a manner similar to that in Example 1, seeds of maize were germinated and 7 g of top portions of the young sheath were collected from seedlings. These were frozen in 10 ml of liquid nitrogen and triturated with a mortar and a pestle to form fine powders. After evaporating liquid nitrogen, RNA was extracted by the conventional manner (guanidine thiocyanate/cesium chloride method) and 1 mg of the total RNA was collected from the extract by ethanol precipitation.

Example 6 (Preparation of an oligo DNA primer and RT-PCR)

A mixture of oligo DNAs expected to encode the partial amino acid sequence determined in Example 4 was synthesized in both sense and antisense orientation.

Specifically, as a nucleotide sequence expected from 8 amino acid residues: Val Ile His Asp Gly Thr Trp Thr in the partial amino acid sequence 2 described in Example 4, a 23-mer in antisense orientation: 5'-GTCCAIGTICC(AG)TC(AG)TGIATIAC-3' (SEQ ID NO:5) was synthesized.

Further, as a nucleotide sequence expected from 8 amino

acid residues: Gly Glu Ala Val Tyr Val Asp Asp in the partial amino acid sequence 4 described in Example 4, a 23-mer in sense orientation: 5'-GGIGA(AG)GCIGTITA(TC)GTIGA(TC)GA-3' (SEQ ID NO:6) was synthesized.

A reverse transcription reaction was carried out using, among them, one synthesized in antisense orientation as a primer and a commercially available reverse transcription PCR kit (RNA-PCR kit, manufactured by Perkin-Elmer Cetus Instruments). Then, the obtained reverse transcription product was subjected again to PCR in which an oligo DNA synthesized in sense orientation was added. As the result, amplification of cDNA fragment was confirmed.

Example 7 (Cloning of the PCR-amplified fragment into a vector and analysis of the structure)

The amplified cDNA fragment obtained in Example 6 was purified and cloned into a plasmid vector pCRII (manufactured by Invitrogen). Further, the nucleotide sequence of the insert in said plasmid vector was determined by 373A DNA Sequencer (manufactured by Applied Biosystems) using ABI PRISM Dye Primer Cycle Sequencing Ready Reaction Kits (manufactured by Applied Biosystems) and the structure of said cDNA fragment was determined. As a result, it was revealed that the cloned cDNA fragment contained 2 kinds having different structure, one corresponding to Nos. 1,839

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to 3,785 nucleotides in the nucleotide sequence shown by SEQ ID NO: ~~2~~ 1 and the other corresponding to Nos. 1,858 to 3,806 nucleotides in the nucleotide sequence shown by SEQ ID NO: ~~4~~ 3.

Example 8 (Isolation of a complete length cDNA clone)

Based on the nucleotide sequence information obtained in Example 7, nucleotide sequences specific for said 2 cDNAs, respectively, were searched and oligo DNAs for the parts were synthesized in sense and antisense orientations.

Specifically, as the sense oligo DNAs corresponding to the nucleotide sequence shown by SEQ ID NO: ~~2~~ 1, two kinds:

a 28-mer: 5'-GCTGGTCAAAATATTGGTGTCGTGATTG-3' (SEQ ID NO:7) (common), and

a 28-mer: 5'-GATTGCTGAAACACAAAGATATGCTAAT-3' (SEQ ID NO:8), and as the antisense oligo DNAs, four kinds:

a 27-mer: 5'-TGGCTGCAGATTTTCTGTGCTATACTC-3' (SEQ ID NO:9) (common),

a 27-mer: 5'-TGCTTTGCAGCCATATTAGCATATCTT-3' (SEQ ID NO:10),

a 24-mer: 5'-ACAGCCTTTTGGAAGCCACCTGGA-3' (SEQ ID NO:11), and

a 24-mer: 5'-ATCGGACTTGTTGTCGGCCTTGAC-3' (SEQ ID NO:12)

were synthesized.

Also, as the sense oligo DNAs corresponding to the nucleotide sequence shown by SEQ ID NO: ~~4~~ 3, two kinds:

a 28-mer: 5'-GCTGGTCAAAATATTGGTGTCGTGATTG-3' (SEQ ID NO:7) (common), and

a 28-mer: 5'-GATTGCTCAAACACAGAAGTATGCCTAC-3' (SEQ ID NO:13), and as the antisense oligo DNAs, three kinds:

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(SEQ ID NO:9)

a 27-mer: 5'-TGGCTGCAGATTTTCTGTGCTATACTC-3' (common),

(SEQ ID NO:14)

a 25-mer: 5'-CTTTGCCGCCATGTAGGCATACTTC-3', and

a 24-mer: 5'-TTCCACCTATGGTTGCAGTGTTCC-3', (SEQ ID NO:15)

were synthesized.

Using them as primers, RACE process was carried out with a commercially available Marathon cDNA Amplification Kit (manufactured by Clontech) to obtain cDNA fragments having terminals in 5'-orientation and 3'-orientation, respectively. Further, a complete length cDNA was obtained by ligating them and cloned into a plasmid vector pCRII (manufactured by Invitrogen).

Example 9 (Analysis of nucleotide sequence and determination of amino acid sequence of cDNA clones)

For two cDNA clones obtained in Example 8, analysis of nucleotide sequence was carried out with 373A DNA Sequencer (manufactured by Applied Biosystem) using ABI PRISM Dye Primer Cycle Sequencing Ready Reaction Kits, Dye Terminator Cycle Sequencing Kits (manufactured by Applied Biosystems). As a result, it was revealed that the genes of the present invention were cDNAs having 4,412 bp and 4,359 bp, respectively (see SEQ ID NOS: ~~2~~ 1 and ~~4~~ 3).

Further, based upon said nucleotide sequence, the total amino acid sequences encoded by the genes of the present invention were determined with GENETYX Gene Analysis

Software (manufactured by SDC, Software Development Co.). It was revealed that they were proteins having 1,358 and 1,349 amino acid residues, respectively (see SEQ ID NOS: ~~1~~ 2 and ~~3~~ 4).

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Example 10 (Construction of aldehyde oxidase expression plasmid for direct introduction)

In order to allow expression of the gene of the present invention derived from maize by introducing in a plant cell, the following direct introduction expression vector for plant, for example, is constructed.

A GUS expression vector pBI221 (manufactured by Clontech) derived from pUC19 is digested by restriction enzymes SmaI and SacI (both being manufactured by Takara Shuzo) and 2.8 Kbp fraction is recovered removing GUS structural gene. The terminus is blunted with T4 DNA polymerase (manufactured by Takara Shuzo). Then, the terminus is treated for de-phosphorylation with bacterial alkaline phosphatase (manufactured by Takara Shuzo).

On the other hand, the complete length cDNA obtained in Example 8 is prepared for an insert gene and the terminus is blunted with T4 DNA polymerase in a similar manner. Afterwards, the both are ligated with T4 DNA ligase (DNA Ligation Kit Ver. 2, manufactured by Takara Shuzo) and used for transforming competent cells of *E. coli* HB101 strain

(manufactured by Takara Shuzo), from which Ampicillin resistant strains are selected. Among the recombinant plasmid amplified from the selected strains, clones in which a coding region for the aldehyde oxidase is inserted in normal orientation in relation to 35S promoter derived from cauliflower mosaic virus and the terminator derived from nopaline synthase and cloned in which said region is inserted in reverse orientation are selected and taken as expression vectors for direct introduction, respectively.

Example 11 (Construction of aldehyde oxidase expression plasmid for indirect introduction)

In order to allow expression of the aldehyde oxidase gene derived from maize by introducing in a plant cell, the following indirect introduction expression vector for plant, for example, is constructed.

In a manner similar to that in Example 10, the aldehyde oxidase gene of which the terminus is blunted is prepared for an insert gene. On the other hand, a GUS expression binary vector pBI121 (manufactured by Clontech) derived from pBIN19 is digested by restriction enzymes SmaI and SacI and a fraction is recovered removing GUS structural gene. The terminus is blunted in a similar manner and treated for de-phosphorylation. The both are ligated and used for transforming *E. coli*. The recombinant plasmid are selected

and taken as aldehyde oxidase expression vectors for indirect introduction. Further, the plasmid vectors are transferred to the strain *Agrobacterium tumefaciens* LBA4404 by the tri-parental method (GUS gene fusion system, manufactured by Clontech).

Example 12 (Creation of a transformed plant by introducing aldehyde oxidase expression plasmid; part 1)

The expression vectors for direct introduction obtainable in Example 10 are introduced by a particle gun into an aseptically cultured immature scutellum of rice plant according to a method described in Shimada et al., Ikushugaku Zasshi, 1994, 44 Supplement 1, 66, to obtain transformed rice plants. Similarly, they are introduced by a particle gun into an aseptically cultured immature scutellum of wheat plant according to a method described in Takumi et al., Ikushugaku Zasshi, 1995, 45 Supplement 1, 57, to obtain transformed wheat plants. Similarly, they are introduced by a particle gun into an aseptically cultured immature scutellum of barley plant according to a method described in Hagio et al., Ikushugaku Zasshi, 1994, 44 Supplement 1, 67, to obtain transformed barley plants. Similarly, they are introduced by particle gun into an adventitious embryo of maize according to a method described in M. E. Fromm et al., Bio/Technology, 1990, 8, 833 - 839, to obtain transformed

maize plants. Further, the expression vectors for direct introduction obtained in Example 10 are introduced by a particle gun into an adventitious embryo of soybean according to a method described in Japanese Patent Application Hei 3-291501 to obtain transformed soybean plants.

Example 13 (Creation of a transformed plant by introducing aldehyde oxidase expression plasmid; part 2)

The strains from *Agrobacterium tumefaciens* LBA4404 into which the aldehyde oxidase expression vectors for indirect introduction are introduced, obtainable in Example 11, are infected to an aseptically cultured leaf of tobacco by a method described in Uchimiya, Manual for Plant Gene Manipulation (Method for Producing Transgenic Plants), Published by Kodansha Scientific (ISBN4-06-153513-7), 1990, pages 27 - 33, to obtain transformed tobacco plants. Similarly, they are infected to a petiole of an aseptically cultured seedling of carrot by a method described in N. Pawlicki et al., Plant Cell, Tissue and Organ Culture, 1992, 31, 129 - 139, to obtain transformed carrot plants. Further, they are infected to a hypocotyl or cotyledon of an aseptically cultured seedling of Lotus corniculatus by a method described in Nagasawa et al., Ikushugaku Zasshi, 1995, 45 Supplement 1, 143, to obtain transformed Lotus corniculatus plants. Similarly, they are infected to an

aseptically cultured adventitious embryo of alfalfa by a method described in R. Desgagnes et al., Plant Cell, Tissue and Organ Culture, 1995, 42, 129 - 140, to obtain transformed alfalfa plants. Similarly, they are infected to an epicotyl or cotyledon of an aseptically cultured seedling of pea by a method described in J. Pounti-Kaerlas et al., Theoretical and Applied Genetics, 1990, 80, 246 - 252, to obtain transformed pea plants.